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THE USE OF AN IRON STAIN FOR THE STUDY OF ALVEOLAR DEVELOPMENT IN THE MOUSE MAMMARY GLAND[1]

BY H. E. RAWLINSON

Abstract

A method is described of using whole mounts of the mouse mammary gland with Gömöri's potassium ferrocyanide – hydrochloric acid mixture for staining iron. Because iron accumulates in the epithelial cells in a granular form, most of the mammary gland tree of the mature nonlactating female mouse can be shown up clearly. The staining reaction is quick and uniform, muscle can be clearly differentiated in microdissection, and alveolar development can be estimated from the amount of iron retention in the nonlactating gland.

Introduction

The accumulation of histologically visible iron in the glandular cells of the nonlactating mammary gland of mature female mice has been pointed out (4, 5). This development is so marked that the well known technique of staining whole mounts can be used, with the glands being stained en masse for iron and cleared without counterstain. This gives a clear picture of most of the gland tree, particularly its peripheral alveolar part. The advantages of the method are: (1) the speed and uniformity in the action of the staining mixture; (2) the ease with which muscle and other extraneous tissues can be dissected away; (3) the sharpness of the picture that results from the color differentiation and the high degree of clearing in the nonepithelial tissues; (4) the facility with which alveolar development can be assessed because of the parallel that exists between it and iron retention.

Materials and Methods

In this study female mice of the dba, C57 Black, and C3H strains were used. They were killed, usually by decapitation, and the hides were removed with the mammary glands on them and fixed in 10% neutral buffered formalin (Lillie, 3) for 18 to 24 hr. The gland pads were then dissected free from the skin using glass seekers made from stirring rods, since it is necessary to avoid iron contamination as far as possible until after staining has been accomplished. The pieces of tissue were then soaked in distilled water for an hour or so to

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remove the fixing fluid and dropped into a few cc. of a fresh mixture of 20% hydrochloric acid and 10% potassium ferrocyanide (Gömöri, 2) for 15 to 30 min. This was followed by thorough washing in distilled water and subsequently in 95% alcohol over a period of 5 to 10 hr., using several changes until the staining mixture was completely eliminated. Then the gland was freed from extraneous tissue under the microscope using metal instruments. Dehydration was continued by immersion for two hours or more each, in absolute alcohol, oil of origanum, and xylol. The gland was then flattened by compression between two pieces of glass with a small metal paper clamp and left in xylol for an hour or so to flatten and then mounted in clarite, gum damar, or piccolyte. The results of such a procedure are shown in the figures. In the actual preparations the sharp blue green of the stained iron against the light unstained background considerably heightens the contrast over the photographic reproduction.

Results

The following observations were made.

Speed and Uniformity of Penetration of Staining Fluid

If the process of staining is followed under the microscope, it can be seen that the terminal arborizations of the glandular tree become outlined in a matter of minutes and the process is complete inside of a half hour. Longer immersion does not result in overstaining but does occasion the random precipitation of small particles of Prussian blue on the surface of the tissue. The stain quickly penetrates into the deepest recesses of the tissue mass, even when there is a considerable amount of muscle present, under which conditions the ordinary haematoxylin staining of whole mounts is difficult and uneven.

Ease of Dissection

It is easy to dissect away extraneous tissue by virtue of the fact that the gland tree is sharply outlined while muscle and other nonglandular structures remain unstained. After the gland has been washed clear of excess staining fluid and brought into 95% alcohol it can be dissected under a binocular microscope using a combination of transmitted and direct light. The bleached muscle appears in sharp contrast to the stained gland and it is possible to separate the two very thoroughly. This is a special advantage in dealing with the axillary mammary glands, which are so closely interwoven with muscle.

Clarity of Gland Picture

In tissues stained by this technique the only material that stains sharply is iron in the form of granules, the background taking on a light-green, transparent tint, due, no doubt, to traces of soluble iron in the tissue fluid. The clearing process leaves the iron-containing parts of the gland tree brilliantly outlined against the light background.

Measure of Alveolar Development

The iron in the nonlactating gland of the mature female mouse is stored mainly in the alveoli and smaller ducts leading from them. As a result the stainable iron is a valuable means of assessing the degree of alveolar development. Fig. 1 shows a resting gland of C57 Black female about 60 weeks of age. The alveoli are small but distinct. Fig. 2 shows a similarly resting gland in a dba mouse of about 50 weeks of age. The difference in alveolar development is readily apparent. A study was made of the mammary glands of 50 mice of each of the following strains: C57 Black (having a very low incidence of spontaneous mammary adenocarcinoma); dba (high incidence); C3H (high incidence). It was quite clear that the extent of alveolar development was correlated with the amount of stainable iron, and the investigation is being extended to see whether the iron accumulation can be used to elucidate the factors that affect the growth and development of alveoli.

In this connection it is interesting to note that the alveolar proliferation of the mammary gland during pregnancy is accompanied by an increased deposition of iron. Fig. 3 shows this increase in iron in the gland in an early stage of pregnancy, while Fig. 4 illustrates an even greater accumulation in a more advanced stage. As Schultz (5) has pointed out, just before the termination of pregnancy and during lactation the iron either disappears or is restricted to a few areas that take the stain diffusely.

Fig. 5 shows a hyperplastic nodule. These nodules have been pointed out by many workers as occurring frequently in strains of mice with a high incidence of spontaneous cancer. By the use of the iron technique, it can be seen that such hyperplastic cells, like the normal ones, accumulate iron, and show up well in the whole mounts stained for iron.

Discussion

It has been pointed out in an earlier paper (4) that the mammary glands of the mouse may be closely related to the apocrine sweat glands found in many animals, including man (1), and that the iron content of the mouse gland may be evidence of that relationship. The present study shows that the iron may be used to effect a quick and efficient whole mount stain and that, since the iron content of the resting gland can mirror the alveolar development, such a technique offers definite possibilities for the study of factors affecting the alveoli of the mammary gland. It is difficult to see what purpose such a wholesale piling up of iron might have in the normal functioning of the gland. The significance of the disappearance of the iron during lactation is also obscure. There is evidence in the blue staining of the material in the lumina of the ducts that the iron passes into the milk secretion in the early stages of lactation. As pointed out by Schultz (5) the iron starts to reappear towards the end of lactation and returns in quantity within a week when lactation ceases either in the normal course of events or as a result of weaning the young.

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EXPLANATION OF FIGURES

FIG. 1. *Photograph ×30 of whole mount of a resting mammary gland of a C57 Black female mouse about 60 weeks of age; stained with Gömöri's potassium ferrocyanide – hydrochloric acid mixture. In the actual preparation the color differentiation considerably heightens the contrast.*

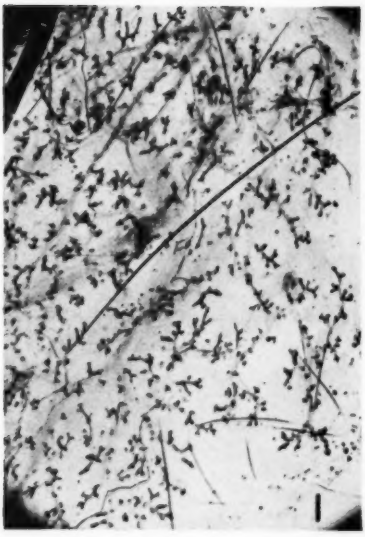
FIG. 2. *Resting gland of a dba female about 50 weeks of age; same treatment and magnification as Fig. 1; more profuse alveolar development shown up by iron stain.*

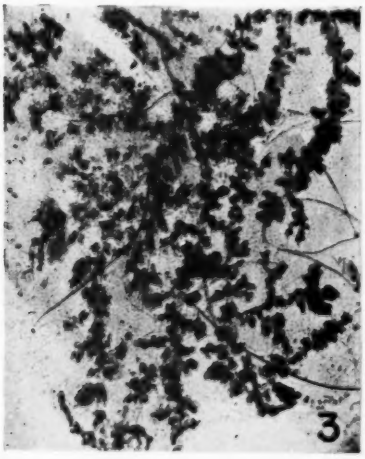
FIG. 3. *Gland of C3H mouse about 25 weeks of age and in early stage of pregnancy; same treatment and magnification as previous figures; shows increase in iron as alveoli develop.*

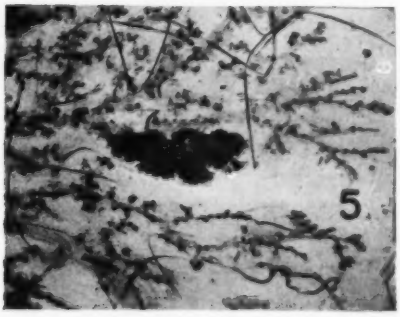
FIG. 4. *Gland of C3H mouse about 25 weeks of age and in later stage of pregnancy; same treatment and magnification as before; shows still further increase in alveolar and iron development.*

FIG. 5. *Gland of a dba mouse about 60 weeks of age; same treatment and magnification as before; shows hyperplastic nodule outlined by the iron stain.*

PLATE I







THE MULTIPLICATION OF INSECT VIRUSES AS ORGANISMS[1]

By G. H. Bergold[2]

Abstract

Electron micrographs of purified preparations of four different insect viruses indicate the presence of morphologically different forms, which are probably stages of multiplication. The virus first appears as a minute spherical body. This body increases in size and the virus appears as an elongated, curved body, surrounded by a membrane. Later the virus particle straightens out, ruptures the membrane, and appears as a rod-shaped particle characteristic of insect viruses. One may assume that the rod-shaped virus particle contains several smaller subunits each of which develops into a rod. The complicated nature of multiplication indicates that insect viruses are organisms with a relatively simple morphological structure of the mature rod.

Introduction

The larvae of many insects are attacked by virus diseases. Two types of polyhedral diseases (2–7, 9–13, 17) and one type of capsule disease (8, 16, 18) have been described.

The polyhedral diseases are so called because of the formation of inclusion bodies, from 1 to 10 μ in diameter, which develop in large numbers in the nuclei of susceptible cells. They are highly refractive, have different and often regular shapes with sharp corners, and consist of protein. In the first type of polyhedral disease i.e. viruses affecting the larvae of susceptible Lepidoptera, polyhedral bodies develop in most tissues with the exception of the gut, the gonads, and the Malpighian tubules (4). In the other type, studied in the larva of the hymenopteran, *Gilpinia hercyniae* (Htg.), the polyhedral bodies develop exclusively in the midgut cells (10–13).

In the capsule disease (8) numerous oval-shaped inclusions are formed, chiefly in the cytoplasm. They consist of protein and are much smaller than polyhedral bodies, measuring about 0.36 by 0.23 μ in the larva of the tortricid *Cacoecia murinana* (Hb.). The nature of the capsules and of the polyhedral bodies from lepidopterous larvae has been studied intensively by physical, chemical, biological, and serological methods (5–9).

The polyhedral bodies and capsules are soluble only in weak alkalies or acids. The alkaline dissolution was chosen for experiments, as the susceptible larvae have an alkaline reaction in the alimentary tract. It was found by studies with the ultracentrifuge and by diffusion measurements that the polyhedral bodies and the capsules consist predominantly of a very homogeneous, noninfectious protein with a molecular weight of 275,000–375,000, depending on the insect species. The infectious virus agent is occluded in the polyhedral bodies and accounts for only a small percentage of their total weight.

[1] *Manuscript received August 10, 1949.*

Contribution No. 2625, Division of Entomology, Science Service, Dominion Department of Agriculture, Ottawa.

[2] *Biologist, Forest Insect Investigations, Laboratory of Insect Pathology, Sault Ste. Marie, Ont., Canada.*

It can be liberated from the polyhedral bodies and the capsules under certain hydrogen ion and salt concentrations. The infectious virus particles are rod-shaped, suspensible in water, and have a high content of desoxyribonucleic acid. The different viruses have somewhat different dimensions: in the gypsy moth, *Porthetria dispar* (L.), 360 by 41 mμ; in the silkworm, *Bombyx mori* (L.), 288 by 40 mμ; in the spruce budworm, *Choristoneura fumiferana* (Clem.), 260 by 28 mμ;* and in the capsule disease of *Cacoecia murinana* (Hb.), 262 by 50 mμ.* There are many virus rods in one polyhedral body but there is only one virus rod in one capsule.

The sedimentation curves of purified virus suspensions obtained in the ultracentrifuge show, in the case of the gypsy moth virus, three different fast-sedimenting components. The silkworm virus and the capsule virus have only one, but a rather wide, sedimentation curve. This means that the virus particles are not homogeneous in size. Early electron micrographs (5) confirmed this assumption. Later and better pictures indicated that some of the virus particles are bundles of two or four single rods (7), which fact explains the nonhomogeneity of the sedimentation curve. Fractionation and tests indicated that the virus activity is associated with the single rods. The highest activity obtained was 4×10^{-13} gm. protein per larva in the silkworm (7). Also observed were peculiar spherical forms and membranelike structures that could not be interpreted (5, 7). The bundle arrangement and the cross structure of the rods were thought to be possibly connected with the multiplication process.

Material and Methods

The virus suspensions were prepared by a method that has been previously described (5–9). Very well purified, dried polyhedral bodies of pure white appearance were the starting material for the virus preparations from the gypsy moth and the silkworm, and purified capsules were used in the case of *Cacoecia murinana*. The material from the spruce budworm was not so pure, but all insoluble impurities were sedimented in five minutes, after alkaline dissolution of the polyhedral bodies, at 6500 r.p.m. (4200g). The virus was sedimented for one hour at 11,000 r.p.m. (12,000g). The clear supernatant of the dissolved polyhedral and capsule protein was discarded, and the virus sediment was suspended in distilled water and again sedimented for one hour at 11,000 r.p.m. The clear supernatant was discarded and the virus sediment suspended and diluted with distilled water to the desired concentration for the electron microscope. Collodion films were used and lowered from a carefully cleaned water surface onto the screens.

Various fixation methods were tried, but the exposure of the small droplets of virus solution on the screens for 10 to 30 min. to vapors of a 2% *osmium tetroxide* solution appeared to be the best (1). No shadow-casting was applied.

* *These figures have to be checked on a greater number of particles.*

The work was carried out with a carefully prepared R.C.A. 50 kv. electron microscope, Type EMU. The instrument was supplied with an objective aperture of about 35 μ in diameter, a self-bias gun, and a telescopic viewing device. All pictures were taken at Step 3 at a magnification of 9500. The exposure time appeared to be important and was measured with a selenium photocell attached on the outside of a side window and a galvanometer with a sensitivity of 5×10^{-10} A per mm. Kodak Spectroscopic plates, Type IV-O, were used. The intensity was kept as low as possible and adjusted with the galvanometer to give an exposure time of three seconds. Focusing was done at the same intensity. The best resolution obtained of the protein material was about 50 Å.

D 11 was used for the development of the negatives, contrast plates and D 72 for the positive contact process. For the negative paper enlargements, Kodabromide F 4 and D 72 were used. The photographic magnification was about five times (from 9500 up to 50,000). This method produces good detail of internal structures, but the outside membranes do not show up so well as on a white background.

Results

Extensive observations with the electron microscope of different preparations of four different viruses led to the discovery of a "life cycle" and of multiplication forms of insect viruses. These are most evident in the polyhedral virus of the silkworm but can be seen also in the other three viruses.

Five stages of the "life-cycle" can be observed. They are summarized in Table I and are shown in Figs. 1 to 98. Figs. 32 and 33, 49, 79 and 80, 97, and 98 are typical pictures showing different stages of development of virus suspensions from *Bombyx mori*, *Porthetria dispar*, *Choristoneura fumiferana*, and *Cacoecia murinana*.

TABLE I

DESCRIPTIONS OF THE DEVELOPING STAGES OF INSECT VIRUSES

Polyhedral viruses			Capsule virus
Bombyx mori	*Choristoneura fumiferana*	*Porthetria dispar*	*Cacoecia murinana*
Stage I			
Somewhat spherical forms developing from unknown size			
200 mμ	300 mμ	300 mμ	150 mμ
Elongated "germ" becoming visible in a spherical membrane			
Some differentiation. Figs. 1 – 6	Some differentiation. Figs. 34 – 38	Clear differentiation. Germ wider. Figs. 51 – 57	Clear differentiation. Figs. 81 – 84

TABLE I—*Concluded*

DESCRIPTION OF THE DEVELOPING STAGES OF INSECT VIRUSES—*Concluded*

<table>
<tr><th colspan="3">Polyhedral viruses</th><th>Capsule virus</th></tr>
<tr><th>Bombyx mori</th><th>Choristoneura fumiferana</th><th>Porthetria dispar</th><th>Cacoecia murinana</th></tr>
<tr><td colspan="4">Stage II
"Germ" elongating bending to a curved shape inside of the membrane</td></tr>
<tr><td>Figs. 7 – 10</td><td>Figs. 39 – 40</td><td>Figs. 58 – 60</td><td>Figs. 85 – 87</td></tr>
<tr><td colspan="4">Stage III
Curve-shaped "germ" straightens out to a more rod-shaped particle (still in membrane)</td></tr>
<tr><td>Figs. 11 – 14</td><td>Fig. 41</td><td>Figs. 61 – 62</td><td>Figs. 88 – 91
Double forms visible</td></tr>
<tr><td colspan="3">Stage IV
Shrinking of the germ and decrease in width (still in membrane)</td><td>Stage IV
No shrinking could be observed</td></tr>
<tr><td>Heavy structural changes.
Figs. 15 – 21</td><td>Sometimes dividing lengthwise.
Figs. 42 – 44</td><td>Heavy structural changes. Dividing lengthwise.
Figs. 63 – 73</td><td></td></tr>
<tr><td colspan="4">Stage V
Rod-shaped particle leaving spherical membrane</td></tr>
<tr><td colspan="2">Second, tube-shaped membrane</td><td rowspan="3">Splitting frequently in four rods.
Figs. 74 – 76</td><td>Fig. 92</td></tr>
<tr><td>Figs. 22 – 24</td><td>Fig. 45</td><td></td></tr>
<tr><td>Empty membranes
Figs. 25 – 27</td><td></td><td></td></tr>
<tr><td colspan="2">Final rod-shaped, smooth particle</td><td rowspan="3">Final rod-shaped, smooth particle.
Figs. 77 – 78</td><td rowspan="3">Final wider rod, slightly bent; with structures.
Figs. 93 – 96</td></tr>
<tr><td>Figs. 28 – 29</td><td>Figs. 48 – 50</td></tr>
<tr><td colspan="2">Sometimes showing groove lengthwise</td></tr>
<tr><td>Figs. 30 – 31</td><td>Figs. 46 – 47</td><td></td><td></td></tr>
</table>

Discussion

The development of the polyhedral bodies is a very rapid process. It is, therefore, probable that virus particles in different stages of their development become suddenly occluded and that they may be liberated by dissolution.

The only other particles that could be occluded by the polyhedral bodies are nuclear components of the host cell. Numerous dissolutions of different polyhedral bodies always show the same forms. This would not result if any nuclear components become occluded.

The next important point is to prove the connection between the infectious rod-shaped particles (Figs. 28 to 31) and the spherical forms. This is demonstrated by Figs. 22 to 24, which show rod-shaped particles that have just emerged from a spherical membrane. Earlier stages (Figs. 4 to 21) also show that the particle is surrounded by a membrane. This membrane returns somewhat to its original spherical form (Figs. 4, 6, 8) when the virus particle escapes (Figs. 22 to 24). The presence of this spherical membrane is therefore the proof of a connection between the virus rods and the spherical developing stages. The early stages (Figs. 1 to 3, 34 to 36, 51 to 52) are not convincing. But one may be able to fractionate and concentrate these and probably also even earlier stages. It seems unlikely (6) that the reported particles of 10 mμ diameter (2, 4, 15) are the initial stages.

Also, Figs. 22 to 26 and 45 show a tube-shaped membrane of the same dimensions as the rods. It appears therefore that the rods are surrounded in the developing stages by two membranes. It is probable that the particles observed in Figs. 22 to 24 were in an advanced stage of development when occluded by the polyhedral protein. The emergence of the rods certainly takes place while the rods are drying out on the film in the high vacuum. Both membranes must consist of a very elastic substance. Figs. 25 to 27 show that the membranes are not artifacts since they break in a characteristic way and have the appearance of a broken rubber ball with curled edges. So far it is uncertain whether the rod leaves the second, tube-shaped membrane under natural conditions.

There is, as yet, no explanation of how the rod-shaped particles begin to multiply again. Two possibilities are suggested: either the rod-shaped particle shrinks enormously to a small sphere, or it contains several small subunits. The first possibility is very unlikely. But the second can be considered, and may be confirmed by the following. If only one rod develops from one, no multiplication takes place at all. In the case of the silkworm polyhedral virus, it is not known whether the rods (Figs. 30, 31) showing lengthwise grooves really split. Furthermore, these double rods are rather rare. The rapid increase of virus particles could hardly be explained, if this were the only kind of multiplication. It is therefore probable that a single rod contains smaller subunits, which can develop into rods. The possibility of the existence of such subunits, based on the serological results of parallel mutations of different strains (14), has been discussed even in the case of tobacco mosaic virus.

The complicated nature of development and of multiplication described above certainly indicates that insect viruses are organisms with spherical developing stages and a rod-shaped stage of relatively simple morphological structure.

Acknowledgment

The author would like to thank F. T. Bird for discussing the problem and for his help in preparing this paper.

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EXPLANATION OF PLATES

PLATE I

FIGS. 1 TO 33. *The developing stages of the polyhedral virus of the silkworm, Bombyx mori (L.). Magnification 42,500×.*

FIGS. 1 TO 6. *Stage I. Spherical forms showing the "germ" within a membrane.*

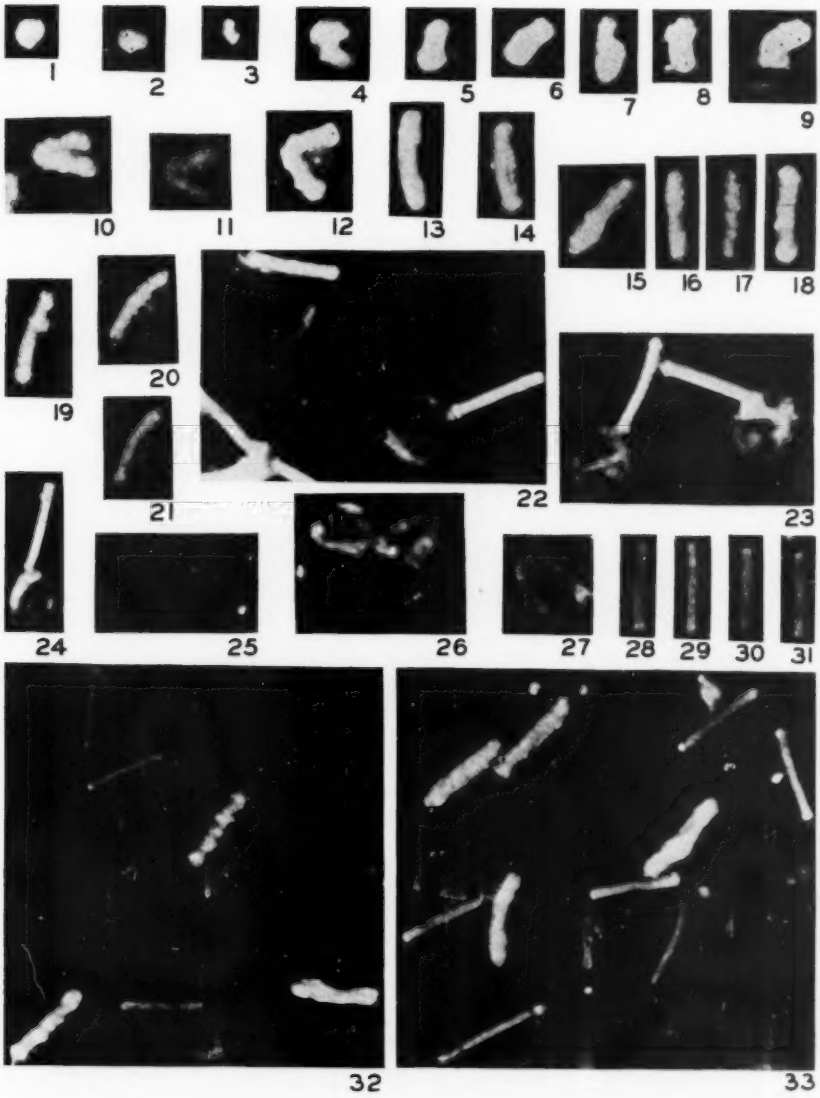
FIGS. 7 TO 10. *Stage II. "Germ" elongating and seen in a curved shape within a membrane.*

FIGS. 11 TO 14. *Stage III. "Germ" straightening out to a rod-shaped form. Still in the membrane.*

FIGS. 15 TO 21. *Stage IV. Shrinking of "germ" and decreasing in width. Heavy structural changes. Still in membrane.*

FIGS. 22 TO 31. *Stage V. Rod-shaped particles leaving spherical and tube-shaped membranes. Figs. 22 to 24; Figs. 25 to 27: empty membranes; Figs. 28, 29: single, rod-shaped, smooth virus particles;* FIGS. 30, 31: *rod-shaped virus particles showing groove lengthwise.*

FIGS. 32, 33. *Typical pictures of virus suspensions from the silkworm, showing different stages of development.*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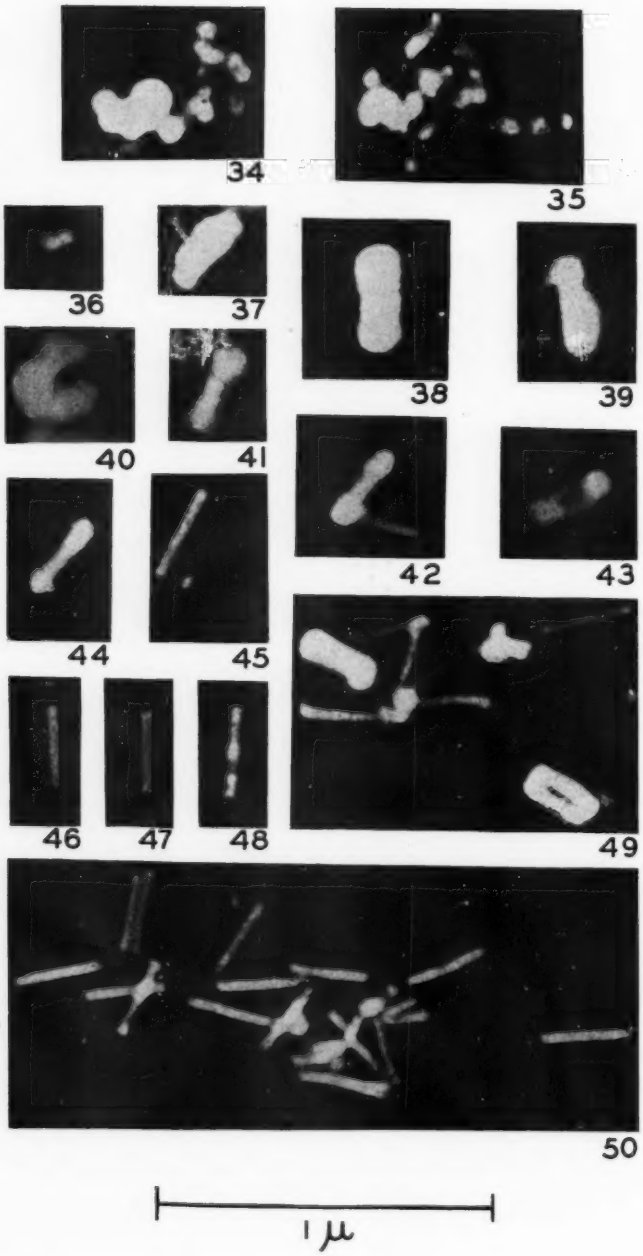
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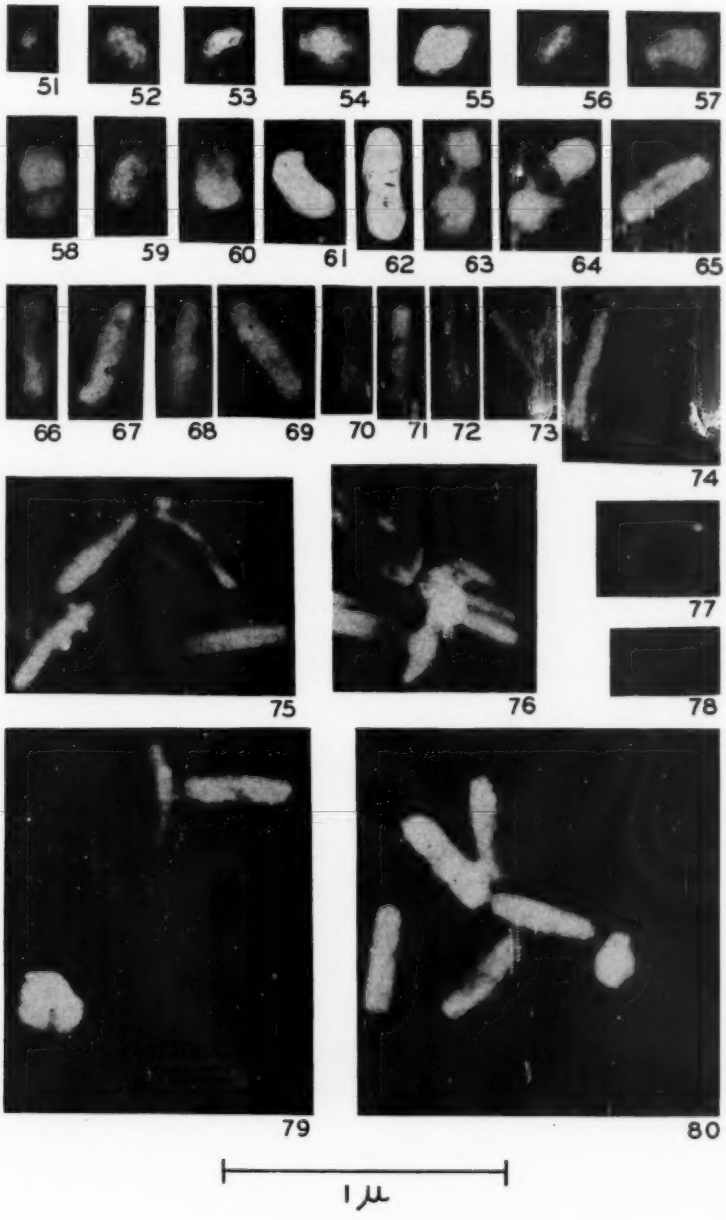
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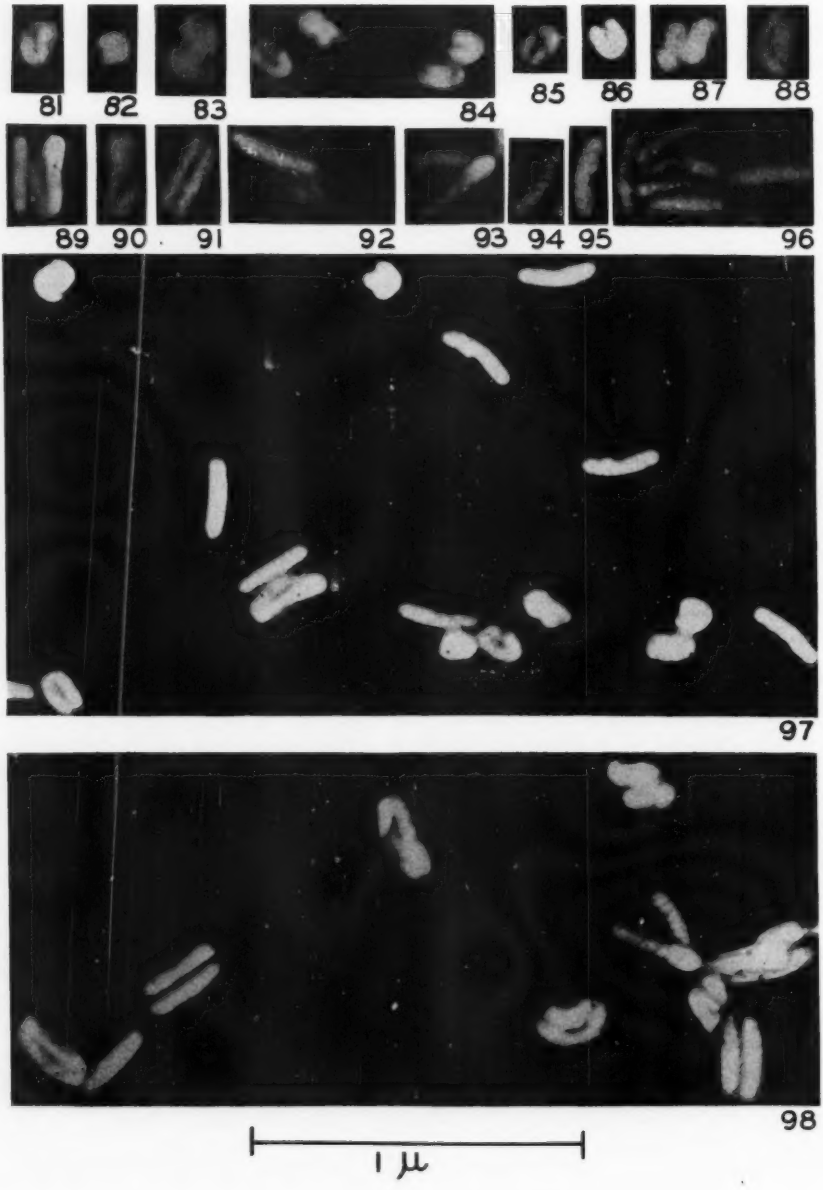
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PLATE II

FIGS. 34 TO 50. *The developing stages of the polyhedral virus of the spruce budworm, Choristoneura fumiferana (Clem.). Magnification 50,000×.*

FIGS. 34 TO 38. *Stage I. Spherical forms showing "germ" within a membrane.*

FIGS. 39, 40. *Stage II. "Germ" elongating and seen in a curved shape within a membrane.*

FIG. 41. *Stage III. "Germ" straightening out to a rod-shaped form. Still in membrane.*

FIGS. 42 TO 44. *Stage IV. Shrinking of "germ" and decreasing in width. Still in membrane. Sometimes dividing lengthwise. Fig. 43.*

FIGS. 45 TO 48, 50. *Stage V. Rod-shaped particles leaving spherical and tube-shaped membranes, Fig. 45; Figs. 46-48, 50: single, rod-shaped, smooth particles; Fig. 48: rod-shaped particle with cross structures; Fig. 47: rod-shaped particle showing groove lengthwise.*

FIG. 49. *Typical picture of virus suspension from the spruce budworm, showing different stages of development.*

PLATE III

FIGS. 51 TO 80. *The developing stages of the polyhedral virus of the gypsy moth, Porthetria dispar (L.). Magnification 42,500×.*

FIGS. 51 TO 57. *Stage I. Spherical forms showing broad "germ" with structures within the membrane.*

FIGS. 58 TO 60. *Stage II. "Germ" elongating and seen in a curved shape within a membrane.*

FIGS. 61, 62. *Stage III. "Germ" straightening out to a rod-shaped form. Still in the membrane.*

FIGS. 63 TO 73. *Stage IV. Shrinking of "germ" and decreasing in width. Heavy structural changes. Sometimes lengthwise divisions visible.*

FIGS. 74 TO 78. *Stage V. Rod-shaped particles leaving spherical membranes. Figs. 74, 75; Figs. 74 to 76: splitting into four single rods; Figs. 77, 78: single, rod-shaped, smooth virus particles.*

FIGS. 79, 80. *Typical pictures of virus suspensions from the gypsy moth, showing different stages of development.*

PLATE IV

FIGS. 81 TO 98. *The developing stages of the capsule virus of Cacoecia murinana (Hb.). Magnification 50,000×.*

FIGS. 81 TO 84. *Stage I. Spherical forms showing the "germ" with structures within the membrane.*

FIGS. 85 TO 87. *Stage II. "Germ" elongating and seen in a curved shape within a membrane.*

FIGS. 88 TO 91. *Stage III. "Germ" straightening out to a rod-shaped form. Double forms visible. Still in the membrane.*

FIGS. 92 TO 96. *Stage V. Rod-shaped particle leaving spherical membrane. Fig. 92; Figs. 93 to 96: single rod-shaped particles broad, slightly bent, with some structures; sometimes in spiral form (Fig. 93).*

FIGS. 97, 98. *Typical pictures of virus suspensions from Cacoecia murinana, showing different stages of development.*

THE PYRIDINE NUCLEOTIDE CONTENT OF HUMAN BLOOD CELLS IN ANEMIA[1]

By M. C. Blanchaer

Abstract

The pyridine nucleotide content of the blood cells was measured in five normal individuals and in 27 hospital patients with various degrees of anemia. The pyridine nucleotide values of 12 anemic patients who had consumed an adequate diet for some time prior to the study were higher than those of the normal subjects. A negative correlation was observed between the cellular pyridine nucleotide content and the severity of the anemia when the latter was expressed as the logarithm of either the red cell count or the hemoglobin concentration. The remaining 15 patients had dietary histories suggestive of a low intake of niacin and protein. The blood cell pyridine nucleotide levels of this group were of the same order as those of the normal subjects but in most cases were distinctly below those of the well nourished anemic patients with a comparable degree of anemia.

Introduction

The realization that nicotinamide is an essential component of the molecule of the two pyridine nucleotides (PN) has led to a number of studies (1, 2, 4, 5, 11, 12) on the relationship of niacin status in man to the pyridine nucleotide content of the blood cells. The neglect of factors other than niacin intake influencing such measurements (5) may have given rise to the conflicting evidence presented in these reports. Vilter *et al.* (11) suggested that anemia per se may affect the PN concentration in the blood cells, since such values were frequently found to be elevated in anemic individuals. Indirect confirmation of this observation may be found in the data of Melnick *et al.* (7) who reported an increased nicotinic acid concentration in the blood cells in anemia.

The present investigation was undertaken to determine the nature of the relationship between anemia and the PN content of the blood cells.

Methods

The subjects consisted of five apparently healthy laboratory workers and 27 hospital patients representing the commoner types of primary and secondary anemia seen in this region. The subjects were divided into two groups on the basis of nutritional status. Group A consisted of the five normal laboratory workers and 12 patients judged to have consumed adequate amounts of a normal diet for some time prior to the study. The remaining patients were assigned to Group B. Most of these had dietary histories suggestive of a low intake of niacin and protein and many suffered from severe illnesses accompanied by general malnutrition, but none showed overt clinical signs of vitamin deficiency.

[1] *Manuscript received October 29, 1949.*

Contribution from the Nutrition Laboratory, Department of Physiology and Medical Research, University of Manitoba, and the Winnipeg General Hospital, Winnipeg, Man.

Blood specimens were obtained by venipuncture approximately three hours after breakfast. The anticoagulant was a dried mixture of ammonium and potassium oxalate (6). Methods described by Wintrobe (13) were used in determining hematocrit values and counting red cells, leucocytes, and reticulocytes. Haemoglobin was estimated by the alkaline hematin method of Clegg and King (3). The PN concentration of each whole blood specimen was determined in duplicate by the fluorometric procedure of Levitas *et al.* (6). Since previous workers (8) using specific biological methods have shown that the pyridine nucleotides in blood are limited to the cells, the whole blood values were corrected for the apparent PN content of the plasma measured by the same fluorometric procedure. No evidence is available to indicate whether the substance in plasma that behaves like the pyridine nucleotides in the fluorometric procedure is limited to the plasma or is present in both plasma and cells. In calculating the results, it was assumed that this substance was limited to the plasma. The corrected values were not markedly different when the alternative assumption was made. The corrected PN levels were assumed to be mainly dependent upon the red cell PN content since it was observed that moderate variations in the leucocyte count had little influence on the values.

Results obtained using an internal standard of N^1-methylnicotinamide agreed closely with those calculated from external standards, suggesting that extraneous material in the samples had little quenching effect on the fluorescence. The average difference between the results of duplicate estimations on 20 specimens of whole blood was 6.4% (S.D. $\pm$ 4.2). The percentage difference between duplicates showed no tendency to vary with the hemoglobin or PN content of the specimens.

Since the degree of anemia may be measured in terms of the hematocrit value, hemoglobin concentration, or red cell count, the PN content of the blood cells was expressed in corresponding units, i.e., micrograms per milliliter cells (PN/ml. cells), micrograms per gram haemoglobin (PN/Hb), and micrograms per billion (10^9) red cells (PN/RBC). The method of calculating the results was as follows:

$$\left.\begin{matrix}\text{Corrected PN/ml.}\\ \text{whole blood}\end{matrix}\right\} = (\mu\text{gm. PN/ml. whole blood}) - \left(\mu\text{gm. PN/ml. plasma} \times \frac{100 - \text{hematocrit}}{100}\right)$$

$$\text{PN/ml. cells} = \frac{\text{Corrected PN/ml. whole blood} \times 100}{\text{Hematocrit}}$$

$$\text{PN/RBC} = \frac{\text{Corrected PN/ml. whole blood}}{\text{Red cells/cu. mm.} \times 10^{-6}}$$

$$\text{PN/Hb} = \frac{\text{Corrected PN/ml. whole blood}}{\text{Gm. hemoglobin/ml. whole blood}}$$

In an attempt to simulate the effect on the method of some of the chemical and physical differences between blood of normal and anemic subjects, the following experiments were performed. Analyses were made on the blood of two normal individuals and compared with the results obtained after dilution with homologous plasma. It may be seen in Table I that addition of plasma to blood had little effect on the final cellular PN values when appropriate corrections were made for dilution and the plasma blank.

TABLE I

THE EFFECT OF DILUTION ON THE ESTIMATION OF PN IN BLOOD CELLS

Subject	Specimen	Hematocrit	Haemoglobin, gm. %	PN*, μgm./ml.	PN/ml. cells	PN/Hb
1	Plasma	—	—	1.4 2.2	—	—
	Blood	47.5	15.8	37.2 39.5	79.0	237
	Blood – plasma	23.6	8.2	20.2 20.2	79.7	229
2	Plasma	—	—	1.0	—	—
	Blood	41.9	13.2	26.2 25.6	60.0	192
	Blood – plasma	16.6	5.6	10.8 11.9	63.9	189

* *Duplicates, uncorrected for plasma blank.*

Results

The results obtained in Group A, which consisted of those subjects who had consumed an adequate diet, are shown in Table II. While the whole blood PN levels of the anemic patients were lower than those of the normal subjects, the cellular PN values were higher. The PN/RBC and PN/Hb values of Group A were found to be negatively related to the logarithm of the red cell count and hemoglobin concentration respectively, as shown in Figs. 1 and 2. A similar but less exact relationship existed between the PN/ml. cells and the hematocrit readings.

In contrast to the findings in Group A, the whole blood PN levels of the subjects in Group B, shown in Table III, decreased roughly in proportion to the severity of the anemia. The cellular PN values were of the same order as those of the normal subjects in Group A or slightly greater. However, as shown in Figs. 1 and 2, the PN/RBC and PN/Hb values with few exceptions, were lower than those of the patients in Group A with comparable degrees of anemia.

TABLE II

Data on subjects in Group A

No.	Sex	Age	W.B.C. per cu. mm.	R.B.C. millions per cu. mm.	Haemoglobin, gm. %	Reticulocytes, %	Hematocrit	Pyridine nucleotides μgm./ml. Plasma	Pyridine nucleotides μgm./ml. Whole blood*	Pyridine nucleotides μgm./ml. Cells	PN/Hb	PN/RBC	Diagnosis
1	M	28	7500		16.4	—	48.5	1.6	35.8	72.1	213	—	Normal
2	M	21	—	—	15.6	—	48.3	1.2	35.5	80.0	194	—	Normal
3	M	24	—	—	15.0	—	44.1	0.3	29.4	66.7	196	—	Normal
4	M	65	9500	5.42	14.1	—	48.5	1.4	31.5	63.5	218	5.68	Normal
5	F	74	6200	5.07	13.7	—	43.6	2.0	33.3	73.8	235	6.35	Bunion
6	F	23	3600	4.92	13.4	—	42.6	2.2	28.5	64.8	206	5.60	Normal
7	F	48	2800	5.05	12.7	0.4	43.0	0.9	31.8	72.3	246	6.20	Iron deficiency anemia; treated
8	M	61	4320	4.74	12.0	—	43.2	1.9	32.5	72.7	262	6.64	Early gastric carcinoma; normal appetite
9	F	66	4100	3.90	11.6	—	38.2	1.2	32.8	84.0	276	8.24	Psychoneurosis
10	F	18	7700	3.20	8.9	4.4	29.4	2.2	32.2	104.2	344	9.60	Abortion with hemorrhage
11	F	67	11,350	3.37	8.4	2.0	27.4	0.7	26.3	94.0	306	7.66	Lung abscess**
12	F	30	10,300	4.35	7.7	1.2	30.3	2.3	31.7	97.2	392	6.91	Iron deficiency anemia
13	F	19	4100	3.24	7.6	0.6	27.0	1.4	25.8	91.4	324	7.63	Duodenal ulcer
14	M	64	8800	2.12	6.9	18.0	25.1	1.7	27.6	104.8	381	12.40	Pernicious anemia; after liver extract
15	F	43	7100	3.85	6.3	1.2	24.2	1.0	20.9	83.1	318	5.22	Iron deficiency anemia
16	F	74	4300	1.81	6.0	18.7	20.4	1.3	21.5	100.0	340	11.28	Pernicious anemia;* after liver extract
17	M	72	6900	3.42	5.2	—	23.7	1.9	24.4	97.1	442	6.73	Bleeding from œsophageal diverticulum

**Uncorrected for plasma blank activity.*

*** After 210 mgm. nicotinamide in five days.*

In neither group was a correlation found between the cellular PN levels and age, sex, plasma protein concentration, leucocyte count, mean corpuscular

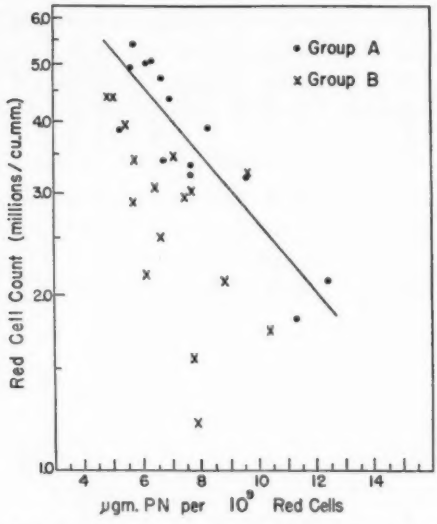

FIG. 1. *The relationship between blood cell PN/RBC values and the logarithm of the whole blood red cell count. The regression line for Group A is shown.*

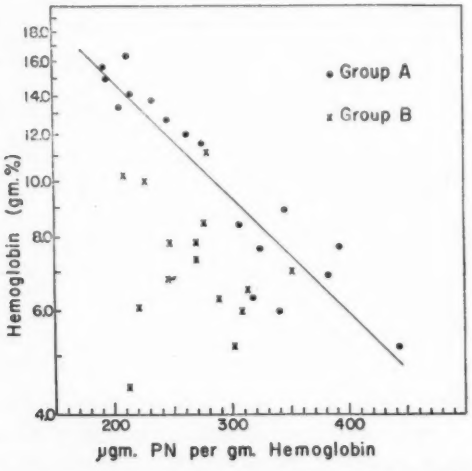

FIG. 2. *The relationship between blood cell PN/Hb values and the logarithm of the whole blood haemoglobin concentration. The regression line for Group A is shown.*

volume, or mean corpuscular haemoglobin concentration. The 16 reticulocyte counts available in the present study were considered too few to permit an

TABLE III

Data on subjects in Group B

No.	Sex	Age	W.B.C. per cu. mm.	R.B.C. millions per cu. mm.	Haemoglobin, gm. %	Reticulocytes, %	Hematocrit	Pyridine nucleotides μgm./ml.			PN/Hb	PN/RBC	Diagnosis
								Plasma	Whole blood*	Cells			
18	M	67	5500	3.25	11.2	—	37.6	1.2	32.6	83.6	280	9.65	Gastrocolic fistula
19	F	45	6500	4.38	10.2	0.6	34.6	1.0	22.1	61.9	210	4.89	Hemorrhage; duodenal ulcer
20	F	88	8400	4.36	10.0	—	34.1	1.4	23.7	67.0	228	5.24	Senile osteoarthritis
21	M	65	4900	3.03	8.5	—	30.2	3.3	25.8	77.9	277	7.75	Bronchopneumonia
22	F	42	12,300	3.40	7.9	1.2	29.4	1.2	20.4	66.8	248	5.77	Lung abscess
23	M	67	8800	3.92	7.9	1.0	28.0	1.1	22.1	76.1	270	5.43	Hemorrhage; duodenal ulcer
24	M	76	5620	3.07	7.3	—	24.2	1.9	21.2	81.4	270	6.41	Diabetes; malnutrition
25	F	87	4200	3.48	7.0	—	28.7	2.0	26.2	86.4	353	7.14	Senile osteoarthritis
26	M	75	3900	2.95	7.0	0.4	26.4	1.4	23.5	83.5	314	7.48	Hemorrhage; gastric carcinoma
27	M	82	10,800	2.52	6.8	1.6	22.5	1.3	17.8	74.7	246	6.67	Hemorrhage; gastric carcinoma
28	F	75	1550	1.74	6.3	0.6	21.0	1.0	19.0	86.8	290	10.48	Pernicious anemia; with diarrhoea
29	F	71	3700	2.17	6.1	3.6	20.2	0.9	14.1	66.3	221	6.17	Ovarian carcinoma; metastases; anorexia
30	M	80	1500	2.09	6.0	—	20.1	1.4	19.6	91.9	310	8.82	Panhypoplastic anemia
31	M	59	8650	2.88	5.2	1.2	22.9	0.9	16.5	68.2	302	5.69	Malfunction of gastroenterostomy
32	M	65	1600	1.20	4.4	0.5	14.8	1.4	10.6	63.9	213	7.89	Sprue; scurvy

* *Uncorrected for plasma blank activity.*

adequate assessment of the influence of an increased number of immature red cells on the cellular PN values.

Discussion

The observations described above suggest that the blood cell PN levels of malnourished anemic subjects cannot be satisfactorily compared with those of normal individuals. It was only when the values of the poorly nourished patients were compared with those of well nourished patients with a similar degree of anemia that a relationship between general nutritional status and the cellular PN levels became apparent. Further observations using more refined methods for assessing niacin status are required before the relationship of blood cell PN values to human niacin sufficiency can be determined.

Because of the apparently normal PN content of the blood cells in pellagra (1, 2, 5), it has been inferred that such measurements are of no use in the detection of niacin deficiency. However, the frequent occurrence of anemia in pellagra (9, 10) would suggest that the observations may have been made in the presence of anemia. The present findings indicate that "normal" blood cell PN levels found under these conditions may reflect a state of niacin deficiency.

Acknowledgments

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THE STABILITY OF ASCORBIC ACID IN SOLUTION[1]

By James Campbell and W. G. Tubb

Abstract

The stability of ascorbic acid in aqueous solution was increased under certain conditions by oxalic acid, metaphosphoric acid, glutathione, thiourea, and sodium diethyldithiocarbamate. A slight protective effect was exerted by creatinine; but formic, phthalic, and orthophosphoric acids, creatine, and caffeine had no demonstrable effect. In all these instances the pH, concentrations of reagents, etc., must be considered. In oxalate and thiourea maximum stability occurred at pH 2.5 to 3.0 and in glutathione at pH 3.6 to 4.2. The latter substance itself was also most stable at pH 3 to 4. At the optimum pH a concentration of 40 m*M* of oxalate gave maximal protection, this being independent of the initial concentration of ascorbic acid over the range 2 to 20 m*M*. Thus a stoichiometric relationship between the concentrations of the ascorbic acid and the oxalate required for protection was not found. A region of minimum solubility of oxalate in water occurred at pH 2.4 to 3.0, which coincides with the pH at which the maximum protective effect occurs and with the highest relative concentration of sodium hydrogen oxalate (or sodium hydrogen oxalate monohydrate). The absorption of ultraviolet light by ascorbic acid was altered by pH, the maximum shifting from 244 to 268 mμ from pH 2.8 to 4.5. The molecular extinction coefficient of ascorbic acid also changed with pH and was minimal at pH 4.0. This effect occurred in oxalate, which has a specific protective effect, and also in formate and orthophosphate, which have no specific protective effect. The possible mechanisms for the protection of ascorbic acid by oxalate are discussed.

Introduction

It is important in the practice of nutrition that ascorbic acid is a highly labile substance. Since the vitamin is apparently maintained in the reduced form in tissues under conditions that would be expected to produce rapid oxidation in the absence of living processes, the problem of its stability is also of physiological interest. While there are many factors that influence the rate of destruction of ascorbic acid, one general aspect of the entire problem concerns the effects of accompanying, nonenzymic, organic substances. Examples of these, which are known to protect the vitamin, are glutathione, cystine, cysteine, and proteins (6, 24). It has been shown that oxalic acid has a powerful protective effect (17, 18, 25) and in the present work particular attention was given to the nature of this phenomenon. Although an array of substances has been studied, it is often difficult to compare their effects on ascorbic acid because of variations in the conditions used by different authors. We have therefore investigated the problem under comparable controlled conditions.

Methods

The water for the preparation of solutions was distilled from a laboratory still and twice from all-glass apparatus. The redistillations lowered the copper content (tested by the method of McFarlane, 21) and improved the

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stability of the ascorbic acid solutions. The citrate–phosphate buffer of McIlvaine (0.1 *M* citric acid and 0.2 *M* disodium phosphate (Clark, 8)) when used, comprised one-half the volume of the final solutions. The pH was determined with a glass electrode (Beckman pH meter).

Ascorbic acid was determined either by titration with iodate (to 5 ml. of the unknown solutions, 1 ml. 10% potassium iodide, 2 ml. of 2 *N* sulphuric acid, and one drop of starch indicator were added and this solution was titrated with 0.01 *N* potassium iodate), or by titration of the solutions adjusted to pH 3.6 with 2,6-dichlorophenolindophenol according to Bessey and King (3). Glutathione in the presence of ascorbic acid was determined as the difference between the iodate and the dye titrations (20, 30). The former titration in the presence of glutathione was done at 50° C., as this improved the sharpness of the end points. To determine oxalate in the presence of ascorbic acid, the former was first separated by precipitation as the calcium salt by adding to 1 ml. of the slightly acid solution in a 15 ml. centrifuge tube, 5 ml. *M*/10 calcium chloride, a drop of methyl red, and ammonia solution to change the indicator to yellow. After centrifuging, the precipitate was dissolved in 2 ml. of 2 *N* sulphuric acid and was titrated with permanganate. The solutions were kept at 37° C. in a dark, electrically-controlled cabinet. At appropriate intervals samples were removed for the determination of ascorbic acid; and by plotting concentration against time, the rates of destruction were determined.

The final concentrations of the constituents of the solutions are given as millimolecular weights per liter (m*M*), except when otherwise mentioned. For example, a solution containing 0.5 gm. oxalic acid per 100 ml. is equivalent to a concentration of 55.5 m*M* oxalate; the proportion of free acid to its salts being dependent chiefly on the final pH of the solutions.

Results

Under conditions that were nearly optimal for stability, the rate of disappearance of ascorbic acid was found to agree with a reaction of the first order, e.g., near the optimum pH for stability in the presence of oxalate, this relation was maintained up to 96 hr. The velocity constant (k) was obtained by the equation:

$$k = 2.303 \frac{(2 - \log c)}{t}$$

where c = the percentage of ascorbic acid remaining

t = time in hours.

Under conditions in which the ascorbic acid was unstable the first order relation was generally not found, and the results of these experiments are expressed as the percentage of ascorbic acid remaining after a certain time interval. In these cases more complicated reactions had probably occurred (Silverblatt, Robinson, and King, 27), and determinations made at shorter time intervals might have revealed a first order relation.

Comparison of the Stability of Ascorbic Acid in Oxalic and Metaphosphoric Acids

In preliminary experiments the stability of 5.68 m*M* ascorbic acid in various concentrations of free oxalic and metaphosphoric acids was determined. Five ml. portions of the solutions were kept at 37° C. in 25 ml. test tubes, and after appropriate intervals were analyzed for ascorbic acid. The ascorbic acid was about as stable in the presence of 375 to 63 m*M* metaphosphoric acid (MPA) from pH 1.65 to 2.18 as in 222 and 55.5 m*M* oxalic acid of pH 1.18 and 1.65 (Table I). When the concentration of oxalic acid was reduced to 11.1 m*M* (pH 2.18) the stability was reduced. Further experiments, *vide infra*, showed that this was due to the lower concentration of oxalic acid and not to the rise in pH.

TABLE I

THE EFFECT OF METAPHOSPHORIC ACID (MPA) AND OF OXALIC ACID AND THEIR SALTS ON THE STABILITY OF ASCORBIC ACID IN SOLUTION. THE INITIAL CONCENTRATION OF ASCORBIC ACID WAS 5.68 m*M*; THE TEMPERATURE 37° C.

Substance added	Concentration, m*M*	pH	*k*
MPA	375	1.65	0.0044
MPA	188	1.88	0.0039
MPA	63	2.18	0.0044
Oxalic acid	222	1.18	0.0039
Oxalic acid	55.5	1.65	0.0039
Oxalic acid	11.1	2.18	0.0057
MPA + sodium hydroxide	188	3.05	0.0094
MPA + sodium phosphate	188	2.97	0.0094
Oxalic acid + sodium hydroxide	55.5	3.45	0.00149
Oxalic acid + sodium phosphate	55.5	3.38	0.00163

When the pH of a 188 m*M* solution of MPA was adjusted to 3.0 ± 0.05, the stability of ascorbic acid was less than in the free MPA (Table I). When the pH of a 55.5 m*M* oxalic acid solution was raised to 3.4 ± 0.05, the protective effect was greater than in the free acid and exceeded that of free MPA. The use of sodium hydroxide or sodium phosphate to adjust the pH of the oxalic acid and MPA solutions produced parallel changes, i.e., the orthophosphate ion had no effect on the stability of the ascorbic acid. The course of the reaction was more regular in the solutions whose pH had been adjusted by alkali. Probably this was due to the buffering effect produced.

The Optimum pH for Stability in Oxalate

The stability of ascorbic acid (initially 5.68 m*M*) in the presence of 55.5 m*M* oxalate over the range pH 1 to 5 was determined at intervals up to 90 hr. (Fig. 1). A definite pH optimum for stability occurred at pH 2.5 to 3.0, and on either side of this optimum the rate of destruction of ascorbic acid

appeared to be equal for equal changes of pH. The rates of disappearance of ascorbic acid followed the first order reaction relationship and the least value of k (0.00075) occurred at pH 2.8.

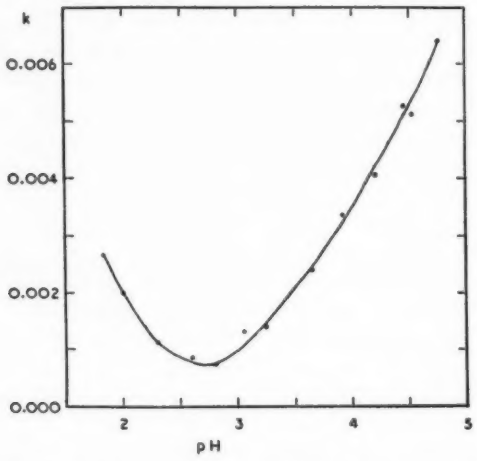

FIG. 1. *The rate of disappearance of ascorbic acid, initial concentration 5.68 mM, from solution in 55.5 mM oxalate at 37° C.*

The Effect of pH on the Solubility of Oxalate

It was observed that alteration of the pH of oxalic acid solutions by the addition of sodium hydroxide had a decided influence on the amount of solid in solution. The nature of this change was determined by adding to 25 ml. of 5% oxalic acid sufficient 1 *N* sodium hydroxide and water to bring to the desired pH and the final volume of 45 ml. When equilibrated at 11.6° C. by continuously rocking the containers in a water bath, solid separated out from the solutions at pH 1.32 to 3.89. Samples were withdrawn after 20 and 46 hr., by means of a pipette with a filter-tip, and the oxalate was determined by the permanganate method. From pH 2.4 to 3.0 (average pH 2.70) the amount of oxalate in solution was minimum (Fig. 2). At pH 2.41 the oxalate per ml. saturated solution was 10.55 mgm. calculated as C_2O_4 (0.1199 *M*). When the temperature was raised to 21.5° C. the minimum solubility of oxalate occurred in the same pH range; i.e., at pH 2.41, 2.65, and 3.00 the amounts of oxalate per ml. saturated solution were 15.4, 15.1, and 15.3 mgm. Thus the range of pH over which the oxalate is least soluble is also the range at which ascorbic acid in solution with lower concentrations of oxalate is most stable.

In solutions similar to the above, but which contained also 5.68 m*M* ascorbic acid, it was found that the added ascorbic acid had little, if any, effect on the solubility of the oxalate under these conditions.

The data of Foote and Andrew (9) on the ternary system sodium oxalate – oxalic acid – water at 25° C. show that the least concentration of oxalate radical in a saturated solution occurs when the saturating solid is sodium

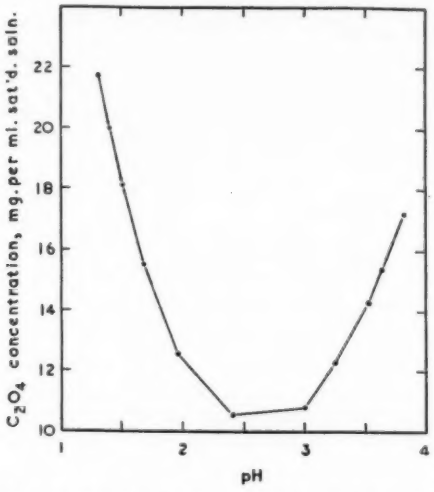

FIG. 2. *The effect of pH on the solubility of oxalate, calculated as C_2O_4, in water at 11.6° C.*

hydrogen oxalate monohydrate and when the composition of the saturated solution can be described in terms of sodium hydrogen oxalate (or sodium hydrogen oxalate monohydrate) and water only, i.e., the system is binary. From our data this least oxalate concentration occurs at pH 2.70.

The dissociation constants of oxalic acid are 1.19 and 4.22. It can be calculated from the Henderson–Hasselbach equation that at pH 2.70 (the mid-point of the range of least solubility of oxalate in mixtures of oxalic acid and sodium oxalate) the ratio oxalic acid/sodium hydrogen oxalate/disodium oxalate is 1/32/1, and that the relative amount of sodium hydrogen oxalate is greatest. In view of the assumptions involved, these calculated values may have limited application. It may be concluded that at the pH of the greatest stability of ascorbic acid in oxalate, the latter is preponderantly in the form sodium hydrogen oxalate (or sodium hydrogen oxalate monohydrate).

The Concentration of Oxalate and the Stability of Ascorbic Acid

The effect of various concentrations of oxalate on the stability of 5.68 mM ascorbic acid was determined at the optimum pH of 2.8 and in the presence of citrate–phosphate buffer, which comprised 50% of the final volume (Table II, Part *b*). The pH of the solutions did not change appreciably during 48 hr. of incubation. The curves drawn from the data showed that as the concentration of oxalate increased, the protective effect increased to a maximum at about 40 mM oxalate and above, giving a value of $k = 0.0006$. Below this

TABLE II

EFFECT OF VARIOUS CONCENTRATIONS OF OXALATE ON THE STABILITY OF ASCORBIC ACID IN THE PRESENCE OF CITRATE–PHOSPHATE BUFFER AT pH 2.8 AND AT 37° C.

Ascorbic acid initial concentration, mM	Concentration of oxalate, mM	pH	k
20	111	2.70	0.000642
	55.5	2.76	0.000936
	27.5	2.78	0.00125
	11.1	2.80	0.00311
	5.55	2.81	0.00415
	2.22	2.81	0.00592
	1.11	2.82	0.00791
	0.0	3.18	0.0206
5.68	111	2.76	0.000601
	55.5	2.77	0.000601
	27.5	2.78	0.00147
	11.1	2.78	0.00346
	5.55	2.80	0.00823
	2.22	2.80	0.0138
	1.11	2.80	0.0146
	0	2.80	0.0206
2.0	111	2.70	0.00121
	55.5	2.76	0.00151
	27.5	2.78	0.00192
	11.1	2.82	0.00521
	5.55	2.82	0.00755
	2.22	2.84	0.0115
	1.11	2.85	0.0173

concentration, k increased with a fairly sharp point of inflection at about 27 mM oxalate. The protective effect was evident, however, even in the lowest oxalate concentrations (1.11 mM). In parallel experiments without buffer it was found that the ascorbic acid was slightly less stable in the presence of the buffer, but this was shown only in the lower concentrations of oxalate and was masked by higher concentrations.

The Stability of Ascorbic Acid in Relation to the Initial Concentration in Oxalate

The effects of varying the concentration of ascorbic acid on its stability in oxalate were determined. The initial concentration of ascorbic acid was varied from 2 to 20 mM and the concentration of oxalate from 1.1 to 111 mM, while the optimum pH was maintained by the use of the citrate–phosphate buffer at one-half strength (Table II, Parts *a*, *b*, and *c*). Over this range of initial concentrations of ascorbic acid the concentration of oxalate required for maximum protection was relatively constant at about 40 mM and was independent, within these limits, of the concentration of ascorbic acid.

This absence of proportionality and the fact that at the low concentration of ascorbic acid the molar ratio of oxalate to it for maximum protection was

1 to 7 (5.68 to 40 mM) shows that a stoichiometric relation is not involved between ascorbic acid and the oxalate required for protection. An increase in the values of k occurred at the lowest concentration of ascorbic acid.

The Effect of Ascorbic Acid on the Stability of Oxalate

Although it was entirely unlikely that the relatively great stability of ascorbic acid in oxalate would be due to preferential oxidation of the latter, this possibility was checked. It was found, as expected, that at the optimum pH for stability of ascorbic acid, the concentration of oxalate remained unaffected.

The Effect of Copper

Many investigators have shown that copper accelerates the oxidation of ascorbic acid (2, 16, 20, 22, 27, and others). In our experiments, the solutions of ascorbic acid, with various concentrations of copper sulphate, were maintained at pH 2.8 by citrate–phosphate buffer. One series of solutions contained 83 mM oxalate, the other, none. Without oxalate, copper at 10^{-4} M concentration or above greatly accelerated the oxidation of ascorbic acid; but at or below 10^{-5} M had much less effect (Table III). This agrees with the findings of Ghosh (10) that copper added in the order of 10^{-6} mole per liter had little effect on the rate of oxidation.

TABLE III

THE EFFECT OF COPPER SULPHATE AND OXALATE ON THE OXIDATION OF ASCORBIC ACID AT 37° C.

Copper concentration	Ascorbic acid after 24 hr. percentage of initial	
	No oxalate	With oxalate, 0.083 M
0	85.5	96.6
$M \times \frac{1}{10^7}$	84.2	96.1
$M \times \frac{1}{10^6}$	82.0	95.9
$M \times \frac{1}{10^5}$	81.0	95.6
$M \times \frac{1}{10^4}$	68.5	95.4
$M \times \frac{1}{10^3}$	59.6	92.1
$M \times \frac{1}{10^2}$	23.6	90.0
$M \times \frac{1}{10}$	5.6	47.2

TABLE IV

THE EFFECTS OF VARIOUS SUBSTANCES ON THE STABILITY OF ASCORBIC ACID (INITIAL CONCENTRATION 5.68 m*M*) IN SOLUTION AT 37° C.

Substance added		pH	Number of solutions in test	McIlvaine buffer	Effect on the disappearance of ascorbic acid
Name	Concentration, m*M*				
Borate	16 to 640	3.3	5	—	Increased destruction
Borate	81	2.48 to 6.91	9	+	Increased destruction
Citrate + phosphate	500 and 100 respectively	Below 4	Large number	+	Slightly increased destruction. Usually about 10% less of the initial ascorbic acid remained after 24 hr.
Phthalate	50	2.2 to 6.4	11	−	No effect
Formate	111	2.42 to 4.50	7	−	No effect
Caffeine	1.3 to 0.16	2.92	3	+	No effect
Creatine	20	4.6	8	+	No effect
Creatinine	20	4.6	9	+	Slightly increased stability
Glutathione	1.1	2.6 to 6.9	9	+	Increased stability, see Fig. 3
Thiourea	0.0001 to 100	3.4 to 2.92	8	−	Increased stability, see Table V
Thiourea	10	2.20 to 6.98	10	+	Increased stability, maximal at pH 2.70, $k = 0.0005$
Diethyldithiocarbamate	0.1 to 10	2.91 to 3.34	4	+	Increased stability, see Table VI

The presence of oxalate inhibited the increased oxidation due to copper up to 10^{-4} *M* concentration. Oxalate also improved the stability in the absence of copper. In solutions with no added copper or oxalate after 24 hr., 85.5% of the initial ascorbic acid remained. When oxalate alone was added, the corresponding value was 96.6%, while the addition of 10^{-5} *M* copper alone decreased the value to 81%. Thus oxalate had a considerable protective effect in the absence of added copper; while copper added (without oxalate) up to 10^{-5} *M* had a relatively small destructive effect. The protective effect of oxalate in the solutions without added copper cannot be explained on the possible removal from the reaction of minute traces of the metal that might contaminate the solutions; for the latter quantity in the solutions prepared with triple distilled water was not far above the limit of sensitivity of the

diethylidithiocarbamate test for copper, which is given as 1 part in 100 million, i.e., 1.6×10^{-7} *M* (5).

The Effects of Various Substances

The effects of a number of substances on the stability of ascorbic acid are summarized in Table IV. Borate was investigated since it can react with certain polyhydric alcohols to form complex compounds (14); but it increased the destruction of ascorbic acid in solution, this being more apparent above pH 3. The citrate and phosphate together slightly accelerated the rate of destruction of ascorbic acid but, for some unknown reason, these substances singly had no noticeable effect below pH 4. In confirmation of the observations of Krishnamurthy and Giri (18), it was found that phthalate, formate, caffeine, and creatine were without effect. Creatinine had some protective influence for at pH 4.6 with 20 m*M* creatinine, and without, the values of *k* were 0.0092 and 0.030 respectively. Glutathione (1.1 m*M*) increased the stability of ascorbic acid (Fig. 3), the maximum occurring at pH 3.5 to 3.9. The glutathione itself was most stable at pH 3 to 4.

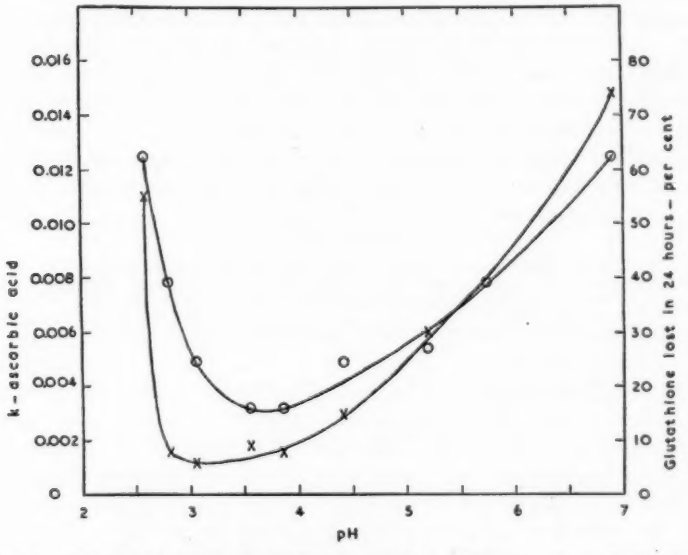

FIG. 3. *The effect of pH on the stability of ascorbic acid* O——O; *and of glutathione* X——X *when mixed in solution with citrate phosphate buffer at 37° C.*

Thiourea in low concentrations has been found to protect ascorbic acid in certain foodstuffs from oxidation (Kawerau and Fearon, 15). In the experiment shown in Table V, this substance in 0.1 m*M* concentration and above increased the stability of ascorbic acid. In another experiment, 10 m*M* thiourea with citrate–phosphate buffer greatly increased the stability from pH 2.20 to 6.98. The maximum stability ($k = 0.0005$) occurred at pH 2.70.

TABLE V

EFFECT OF THIOUREA ON THE STABILITY OF ASCORBIC ACID. INITIAL CONCENTRATION OF ASCORBIC ACID 5.68 mM; TEMPERATURE 37° C.

Thiourea concentration, mM	pH		k
	Initial	After 48 hr.	
100	3.44	3.40	0.00096
10	3.41	3.38	0.00147
1	3.41	3.36	0.00230
0.1	3.41	3.31	0.00575
0.01	3.41	2.90	0.0218
0.001	3.42	2.90	0.0221
0.0001	3.42	2.92	0.0225
0	3.46	2.92	0.0230

McFarlane (22) found that the addition of sodium diethyldithiocarbamate alone or with α,α'-dipyridyl protected ascorbic acid from the accelerated oxidation due to copper and iron. In the present study, pH 3.1 ± 0.2 was maintained with citrate–phosphate buffer and the water was triple distilled, as usual. The diethyldithiocarbamate increased the stability of ascorbic acid under these conditions, to the extent that at 10 mM concentration, k = 0.00059 (Table VI). This indicates that the substance can inhibit the uncatalyzed oxidation of ascorbic acid as well as that catalyzed by heavy metals. In these acid solutions some decomposition of the additive occurred, giving rise to a white turbidity and a disagreeable odor of carbon disulphide.

TABLE VI

EFFECT OF SODIUM DIETHYLDITHIOCARBAMATE ON THE STABILITY OF ASCORBIC ACID IN PRESENCE OF CITRATE–PHOSPHATE BUFFER. INITIAL CONCENTRATION OF ASCORBIC ACID 5.68 mM

Sodium diethyl-dithiocarbamate concentration, mM	pH	k
10	3.34	0.00059
1	3.00	0.0023
0.1	2.91	0.0035
0	2.94	0.0063

Spectral Absorption Characteristics of Ascorbic Acid in Oxalate

The previous experiments indicated that oxalate might protect by depressing the activity of the "enediol" group of ascorbic acid. Since the characteristic absorption peak of ascorbic acid in the ultraviolet can be attributed to this group (11), the effect of oxalate on the spectral absorption of ascorbic acid was determined. A Beckman Spectrophotometer (Cary and Beckman, 7) was used.

In a 5.5 m*M* solution, oxalate alone absorbed at wave lengths below 230 mμ and, as increasing concentrations produced greater absorption in the higher wave lengths and thus obscured the peak due to added ascorbic acid, this was the highest concentration of oxalate that could be used. Increasing the pH of the oxalate solutions from 3.33 to 4.51 caused a slight shift of the absorption curve due to this substance to lower wave lengths (Fig. 4).

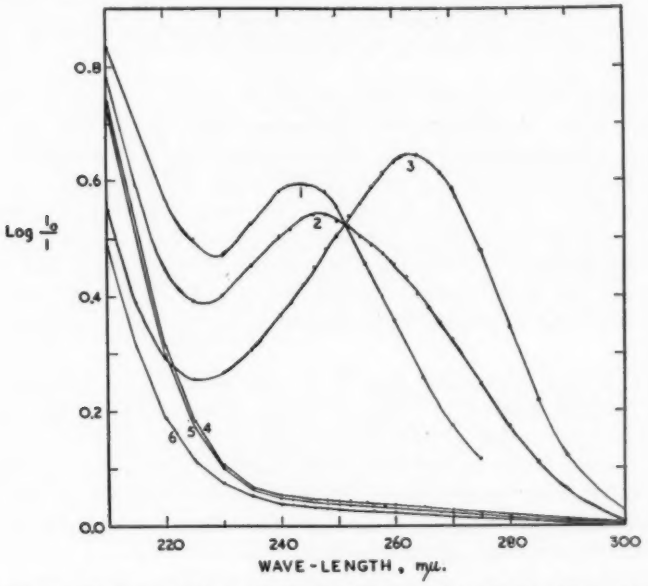

FIG. 4. *The spectral absorption by solutions containing 5.68 mM ascorbic acid in 5.5 mM oxalate at pH 3.33, 3.88, and 4.51, Curves 1, 2, and 3 respectively; and by solutions of 5.5 mM oxalate at pH 3.33, 3.88, and 4.51, Curves 4, 5, and 6 respectively. l = 1 cm.*

Baird *et al.* (1) found that the wave length at which maximum absorption (λ max.) of ultraviolet light by ascorbic acid occurred was influenced by the concentration of hydrochloric acid added. This effect occurred also in our experiments in which other acids were used. In the presence of oxalate the λ max. for ascorbic acid shifted from 244 to 263 mμ when the pH was changed from 3.33 to 4.51 (Fig. 4), and similar shifts of λ max. were observed in formic acid and in orthophosphoric acid.

It was found that the molecular extinction coefficient (ϵ max. as defined by Morton, 23) of ascorbic acid also varied with pH, being minimal at pH 4.0. This occurred in the presence of oxalate, formate, and orthophosphate solutions, the minimal values of log ϵ max., ascorbic acid, at pH 4.0 being 3.946 and 3.916 in oxalate and formate respectively. It is interesting that the molecular absorption of ultraviolet light by ascorbic acid is least near the pH of maximum stability; on the other hand, this occurs in the presence of formate

and phosphate, which have no specific protective effect other than through reduction of the pH, as well as in oxalate, which has.

Discussion

There is an optimum range of pH for the stability of ascorbic acid in solution, which is dependent, at least in part, on the nature of the accompanying substance. In oxalate and in thiourea the optimum range is pH 2.5 to 3.0 while in glutathione it is about pH 3.9. The latter substance is itself most stable from pH 3 to 4.

Oxalate has a strong protective effect on ascorbic acid. In 55 m*M* oxalate at pH 2.8 and 37° C. the value of k for the disappearance of ascorbic acid was about 0.0006. The values of k in 10 m*M* thiourea at pH 2.7 and in 10 m*M* diethyldithiocarbamate at pH 3.3 were 0.0005 and 0.0006 respectively. In 1.07 m*M* glutathione at pH 3.9, $k = 0.003$; but increased concentrations of this substance would most likely increase the protection. Confirming the work of Krishnamurthy and Giri (18) it was found that creatinine had some protective effect but that creatine, formic acid, and phthalic acid had none. The latter result shows that the protective effect is not common to dicarboxylic acids.

There are a number of possible ways by which a substance may inhibit the oxidation of ascorbic acid. The evidence on the protective effect of oxalate may be examined in relation to the possible modes of action of this substance. Borsook, Davenport, Jeffreys, and Warner (4) and Rosenfeld (26) have shown that when oxidation has proceeded to the stage of the formation of oxalate from ascorbic acid, the reaction is irreversible. Thus oxalate cannot be expected to inhibit oxidation by a mass effect.

The evidence indicates that oxalate does not combine chemically with ascorbic acid to form a new compound that is more stable. The concentration of oxalate required for maximum protection was found to be substantially constant and to be independent of the initial concentration of ascorbic acid. The protective effect was present, though submaximal, in low concentrations of oxalate. There was no evidence for a stoichiometric relation between the ascorbic acid and the oxalate required for protection.

An inhibitor of the oxidation of ascorbic acid might act as a hydrogen donor, either reducing dehydroascorbic acid or being oxidized, preferentially, more rapidly. The work of Hopkins and Morgan (12) indicates that glutathione acts in the former way to protect ascorbic acid. Krishnamurthy and Giri (18) failed to obtain reduction of dehydroascorbic acid with oxalic acid, and the observation in these experiments that oxalate at pH 2.8 is stable in the presence of ascorbic acid also relates to this. Apparently, therefore, oxalate does not act as a hydrogen donor to protect ascorbic acid.

It is possible that oxalate may protect by removing the catalytic effect of traces of heavy metals. There is a suggestion of this in the results of the present experiments. It is strongly indicated that oxalate protects ascorbic

acid from oxidation in some other way also, for protection occurred when the effect of heavy metals was negligible. This agrees with the results of Ghosh (10) and Krishnamurthy (17).

From pH 2.4 to 3.0 the solubility of oxalate in mixtures of oxalic acid – sodium oxalate is least; and also the stability of ascorbic acid in lesser concentrations of oxalate is maximum. From the data of Foote and Andrew (9) it seems clear that under these conditions the amounts of the monosodium salt in solution are very large in relation to the amounts of free acid and disodium salt. The correspondence of the maximum stability of ascorbic acid with the highest relative concentration of sodium hydrogen oxalate (or sodium hydrogen oxalate monohydrate) cannot be explained by the effect of pH alone because the maximum stability occurs at a different pH in the presence of glutathione and the effect of pH is apparently not the same in metaphosphoric acid solution (Table I). The optimum pH for stability in thiourea, however, is about the same as in oxalate.

The observation that the addition of ascorbic acid did not alter the solubility of oxalate does not support the possibility that a complex of the two substances may be formed, but to test this properly it would be necessary to use higher concentrations of ascorbic acid.

The ionization constants, pK_1 and pK_2, of ascorbic acid are given as 4.12 and 11.51 at 22° to 23° C. by Kumler and Daniels (19), so that the optimum effect of oxalate is exerted on the (undissociated) acid form of the vitamin. The hypothesis of Weissberger and LuValle (28) and of Weissberger, LuValle, and Thomas (29) apparently does not cover this pronounced effect.

The "enediol" group of the ascorbic acid molecule is the site of the first step in its oxidation and is responsible for its absorption of ultraviolet light. The pH of the solutions influences not only the wave length at which maximum absorption by ascorbic acid occurs, but also the extent of the absorption, which is minimal at pH 4. Thus the absorption is minimal near the pH of maximum stability. The effect appears to be dependent chiefly on pH, since it occurred in the presence of oxalate, formate, and orthophosphate ions. Of these, oxalate has a specific protective effect against oxidation of the ascorbic acid while the formate and orthophosphate have not. Thus the changes in ultraviolet absorption do not reveal a specific effect of oxalate on the "enediol" group.

The evidence available on the nature of the protection afforded by oxalate appears to eliminate the first four possibilities and leaves the open question: Is ascorbic acid protected by an orientation of oxalate in solution with it so as to cause a depression of the activity of the "enediol" group?

The practical considerations arising from these investigations can be briefly discussed. The ascorbic acid content of citrus fruits is of the order of 2 mM, which is within the range of concentrations investigated (Table II). From the discussion of Jeghers and Murphy (13) it is strongly indicated that the toxicity of oxalate is too high to permit of its addition to foodstuffs. The use of oxalate

as an anticoagulant for blood should, in the presence of acid protein precipitants, have a protective influence on the blood ascorbic acid. The studies have shown, in agreement with those of previous workers, that the stability of ascorbic acid is favored by an acid pH, the absence of copper, and the presence of certain protective substances.

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